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Evaluation of Biomarkers in Patients with Different Periodontal Statuses

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Abstract

Background: The aim of this study was to compare the levels of salivary nuclear factor kappa-β receptor activator ligand (RANKL), osteoprotegerin (OPG), tumor necrosis factor alpha (TNF)-α, and sclerostin (SOST) in individuals with different periodontal statuses and to examine their relationship with periodontal status.

Methods: A total of 60 individuals, 20 with periodontal health (S), 20 with gingivitis (G), and 20 with periodontitis (P) were included in this study. Periodontal parameters and saliva samples were recorded from the subjects. Tumor necrosis factor alpha, OPG, RANKL, and SOST levels in saliva samples were determined by enzyme-linked immunosorbent assay (ELISA). The Shapiro-Wilk test, Mann–Whitney U-test, Kruskal–Wallis test, and Chi-square test were used for statistical analysis of the samples.

Results: The mean periodontal parameters and TNF-α, SOST, and RANKL levels of individuals with periodontitis were significantly higher than those of gingivitis and periodontally healthy individuals ($P < .05$). Although periodontal parameters and TNF-α levels showed statistically significant differences between gingivitis and periodontal healthy individuals, there was no difference between the 2 groups in terms of RANKL, OPG, and SOST levels ($P > .05$).

Conclusion: The identification of biomarkers that play a role in bone metabolism in periodontal disease provides a better understanding of the pathophysiological mechanism of the disease. Increased SOST levels in saliva in individuals with periodontitis indicated that SOST may be a diagnostically accurate biomarker in evaluating bone metabolism in periodontal disease.

Keywords: Bone marker, cytokine, inflammatory, periodontal disease, WNT pathway

INTRODUCTION

Periodontal disease is an inflammatory disease with a multifactorial etiology. In the beginning and progression of the abovementioned disease, microbial dental plaque constitutes the primary etiologic factor. Plaque microorganisms and their products directly trigger periodontal disease, and the interaction between the host immune response and periodontal pathogens determines the characteristics of periodontal destruction.[1,3] The inflammatory process that drives bone destruction in periodontitis is a complex network of signal transduction pathways influenced by several systemic and local variables, including cells that maintain bone homeostasis, inflammatory infiltrates, and cytokines that help regulate bone metabolism, hormones, chemokines, and biochemical stimulation.[4,5] Hence, comprehending the relationship between bone formation and the inflammatory process in the tissue is essential to better understand periodontal disease and provide its treatment.

Bone metabolism has been the subject of many studies from the past to the present. The WNT (Wingless and INT-1)/β-catenin pathway plays an essential role in regulating bone metabolism.[6] WNT signaling pathway regulates bone metabolism. This pathway includes a broad family of secreted signaling glycoproteins, where WNT proteins regulate apoptosis, cell differentiation, proliferation, cell survival, polarity, and migration in various cell types. Wingless and INT-1 signaling expresses

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genes important for the differentiation, proliferation, activity, and maturation of osteoblast precursor cells.[7] Sclerostin (SOST) significantly regulates WNT signaling in bone metabolism.[8] Sclerostin reduces osteoblastogenesis and osteoblastic activity, which antagonizes the WNT signaling pathway.[9] Sclerostin may also promote bone resorption and osteoclast differentiation by activating the "receptor activator of NF-κB ligand" (RANKL).[10] Moreover, it can increase the expression of inflammation-related genes and intracellular reagents.[11] The WNT gene has been associated with bone resorption in severe chronic periodontitis, as reported by Divaris et al.[12] Several studies have shown that blocking the SOST gene or its function, which is crucial for bone metabolism, can repair bone defects in periodontitis models.[13] Furthermore, SOST levels were elevated in humans with chronic periodontitis compared with healthy people; hence, SOST has been suggested as promising candidate biomarker, as well as pharmacotherapeutic target.[14]

Tumor necrosis factor (TNF)-α represents a proinflammatory cytokine, and its expression in periodontal tissues increases in periodontal disease. TNF-α increases the response of the host to bacteria and induces resorption in periodontal disease by stimulating bone.[15] It has been demonstrated that injecting TNF-α antagonists decreases the inflammatory cell count in the alveolar bone.[16] In a mouse model of osteoporosis, TNF-α stimulates SOST expression under inflammatory conditions.[17] However, studies examining TNF-α's role in WNT signaling upregulation in chronic periodontitis have not been conducted. A high prevalence of periodontal disease affects most adult individuals worldwide. The economic burden associated with periodontitis treatment emphasizes the significance of applying novel diagnostic and therapeutic interventions.[18] The central pathological process of periodontitis is characterized by inflammatory osteoclastogenesis, which is mediated by proinflammatory mediators. Hence, periodontal bone destruction indicators can be employed to predict disease activity and ensure that clinicians identify patients with high susceptibility or sites with a high risk for disease progression.[19]

The diagnosis of periodontitis typically involves clinical measurements to assess the presence of inflammation and radiographic evaluation of the alveolar bone.[13] An ideal diagnostic tool should offer precise data regarding the existence and intensity of the disease, as well as the ability to forecast future disease status and activity.[15] Saliva is utilized to quantitatively evaluate host-derived molecules at specific sites in a dependable and less prejudiced manner.[16] An analysis of sites with varying levels of periodontal health can be used to identify a biomolecule as a diagnostic biomarker. This approach is commonly employed as an initial step in comprehending the functions of molecules in bone metabolism and the periodontal inflammatory process. The aim of this study was to identify the expression profiles of SOST, RANKL/OPG, and TNF-α in saliva samples of periodontally healthy individuals, individuals with gingivitis, and individuals with periodontitis.

MATERIAL AND METHODS

Our study was conducted in accordance with the STROBE protocol.

Study Population

Sixty patients (16 men and 44 women; aged in the range of 25-49 years; with an average age of 34.83-7.37 years), who presented to Necmettin Erbakan University, Faculty of Dentistry, Department of Periodontology, were enrolled in this research. Written informed consent was obtained from all individuals, and approval for the study protocol was obtained from the Ethics Committee of Necmettin Erbakan University (Approval no: 2021/05-62, Date: May 11, 2021). The current research was registered at ClinicalTrials.gov as NCT05702892.

Periodontal Examinations and Intra-examiner Reproducibility

Probing on debth (PD) and clinical attachment loss (CAL) measurements were performed, and a periodontal probe was used to record the plaque index (PI), gingival index (GI), and bleeding on probing (BoP) scores. The same researcher (F.U.Y.) masked according to the study design and conducted all clinical examinations (at 6 various sites around every tooth: distobuccal, mesiobuccal, mid-buccal, disto-lingual, mesio-lingual, and mid-lingual), group assignments, sampling site selections, and saliva collection.

Before the actual measurement, 10 people were chosen randomly and used for calibrating the researcher. The researcher assessed the subjects in 2 different situations at a 48-hour interval. The researcher's measurements were accepted as adequately reproducible in cases where the measurements obtained at baseline and 48 hour did not differ by more than 10% at the millimeter level.

Exclusion and Inclusion Criteria

The World Workshop for the Classification of Periodontal Diseases and Conditions constituted the basis for the classification of periodontal status.[20] The chosen subjects had at least 20 natural teeth, except for the third molars. Full mouth periodontal evaluation (involving PI, GI, CAL, and PD)[21] and radiography were performed in all subjects.[22]

Gingival index was measured by applying moderate axial pressure and a 45° angle to the probe while engaging 1-2 mm of the gingival margin. The distance between the gingival margin and the most apical part of the sulcus or pocket was used to calculate PD. The distance between the cemento-enamel junction and the sulcus/pocket's most apical aspect was measured, and this was known as CAL. Measurements of PD and CAL were rounded to the nearest full millimeter. We made interproximal recordings as close the contact site as possible. Bleeding on probing recordings were made up to 15 seconds after PD recordings. Clinical parameters were evaluated at 6 sites per tooth (mesiobuccal, buccal, distobuccal, mesiolingual, lingual, and distolingual) using a periodontal probe and included all teeth except the third molars.

Subjects were divided into 3 groups: (1) individuals with clinically healthy periodontium (group 1; n=20; 4 men and 16 women), (2) individuals with gingivitis (group 2; n=20; 4 men and 16 women), and (3) individuals with periodontitis (group 3; n=20; 8 men and 12 women).

Study groups were selected based on clinical and radiological examinations, with a diagnosis of gingivitis in individuals with bleeding on probing in ≥10% of all sites, a probing pocket depth ≤3 mm, and no clinical attachment loss or alveolar bone loss. Individuals without bleeding on probing, with no clinical and radiographic attachment loss, and a probing pocket depth of ≤3 mm were considered periodontally healthy subjects (control group) and were enrolled in the study. The inclusion criteria for the periodontitis group were as follows: clinical signs of inflammation on clinical and radiographic examination (swelling and red of the gingival margin), GI (score 2), PD and CAL ≥5 mm, and bone loss impacting >30% of the available teeth. Table 1 summarizes the study population's descriptive statistics.

The exclusion criteria were identified as follows:
(1) Periapical pathologies; (2) systemic diseases (such as diabetes mellitus, cardiovascular diseases, bone-related diseases, and collagen-metabolic disorders or diseases); (3) excessive forces, involving mechanical forces from occlusion and orthodontics; (4) immunosuppressive therapy or radiation; (5) chronic steroid therapy; immunosuppressive therapy or radiation; (6) breastfeeding or pregnancy; (7) smoking in the last five years; or (8) sensitivity or allergy to a medication. There was no history of periodontal treatment or medication use (such as antibiotics, anti-inflammatories, or any other pharmacologic therapy) for a minimum of 6 months in the study participants.

Collection and Evaluation of Saliva Samples
One hour before the unstimulated saliva samples were taken, the patients were asked not to take any nutrients or drugs, not to use cosmetics such as lipstick, and not to perform oral care the night before. The unstimulated saliva samples of the individuals were collected between 09:00 and 11:00 in the morning using the spitting method into polypropylene Eppendorf tubes given to the subjects in the sitting position while they were calm. Blood-contaminated samples were excluded from the study, and sampling was repeated. Centrifugation of saliva samples collected in Eppendorf tubes was performed at 3.220 rpm for 10 minutes. The samples were numbered, placed in spores, and kept at a temperature of −20°C until the analysis was conducted. Tumor necrosis factor alpha, SOST, OPG, or RANKL levels in the collected saliva specimens were determined by ELISA following the instructions of the manufacturer (Elabscience Biotechnology Co., Wuhan, China). The results were calculated as ng/mL by plotting the absorbance-conc entration curve.

Table 1. Study Population

	Periodontal Health	Gingivitis	Periodontitis	*P*
Age	33.10 ± 1.76	31.70 ± 1.63	38.70 ± 1.14[b]	.013
Gender				
Female	16 (36.4)	16 (36.4)	12 (27.3)	.256
Male	4 (25.0)	4 (25.0)	8 (50.0)	

Comparisons among the study groups were performed using the Kruskal–Wallis test followed by the post-hoc Bonferroni-corrected Mann–Whitney U-test. Data are expressed as mean ± SD. [a] [b]Significantly different from Gingivitis group.

Statistical Analysis
Data evaluation was performed using the Statistical Package for Social Sciences version 21.0 software (IBM Corp.; Armonk, NY, USA). Data were summarized as mean and standard deviation, and the Kolmogorov-Smirnov test was conducted to reveal whether the data were normally distributed. Because the data were not normally distributed, the Kruskal-Wallis and Mann-Whitney U-tests were performed to analyze differences in parameters between the groups. The relationships between biomarkers and periodontal data were analyzed using the Spearman correlation coefficient. The sensitivity and specificity of salivary biomarkers in distinguishing patients with periodontitis from periodontally healthy individuals and patients with gingivitis were evaluated using the area under the curve (AUC) and receiver operating characteristic (ROC) curves.

The results were accepted as significant at a level of $P < .05$.

RESULTS

60 individuals, including 20 periodontally healthy individuals (16 women and 4 men), 20 individuals with gingivitis (16 women and 4 men), and 20 subjects with periodontitis (12 women and 8 men), were enrolled in the study. When the average ages of periodontally healthy individuals and individuals with gingivitis were evaluated, the 2 groups did not differ significantly, whereas the average age of individuals with periodontitis was higher than that of the other groups ($P < .05$). However, the groups did not differ significantly about sex ($P = .256$).

Table 2 presents the periodontal evaluations of the subjects enrolled in the study. Individuals with periodontitis had higher PD, CAL, PI, and GI values than those with gingivitis and periodontally healthy individuals. It was determined that BOP values were similar in individuals with periodontitis and gingivitis ($P > .05$), and these values were higher in subjects with periodontal disease than in healthy individuals.

Figure 1 shows the biochemical analysis of the saliva samples. When salivary TNF-α, RANKL, and SOST levels were assessed

Table 2. Periodontal Parameters in the Study Groups

	Periodontal Health	Gingivitis	Periodontitis	P
CAL	-	-	3.34 ± 0.10	
PD	1.50 ± 0.03	1.82 ± 0.03[a]	3.01 ± 0.14[ab]	<.001
PI	0.23 ± 0.05	1.11 ± 0.08[a]	1.55 ± 0.13[ab]	<.001
GI	0.18 ± 0.04	1.06 ± 0.07[a]	1.64 ± 0.12[ab]	<.001
BOP	5.82 ± 0.69	55.05 ± 3.04[a]	71.13 ± 5.32[a]	<.001

Data are expressed as mean ± SD. Comparisons among the study groups were performed using the Kruskal–Wallis test followed by the post-hoc Bonferroni-corrected Mann–Whitney U-test. BoP, bleeding on probing; CAL, clinical attachment loss; GI, gingival index; PD, probing depth; PI, plaque index. [a]Significantly higher than Periodontal healtyh group. [b]Significantly different from Gingivitis group. [ab]Significantly different from the Periodontal healthy and Gingivitis groups. Periodontitis group exhibited the highest clinical values when compared to the other groups (P < .05).

in individuals with gingivitis and periodontally healthy individuals, no significant difference was detected between the groups ($P > .05$), whereas subjects with periodontitis had higher TNF-α, RANKL, and SOST levels ($P < .05$). While salivary OPG levels did not differ significantly between periodontally healthy subjects and subjects with gingivitis ($P > .05$), lower salivary OPG levels were observed in subjects with periodontitis than in other subjects ($P < .05$).

When evaluating the correlation coefficients, there was a statistically significant positive relationship between periodontal parameters (including, CAL and PD) and SOST, TNF-α, RANKL, RANKL/OPG ratios, and SOST/OPG ratios ($P < .05$). However, no correlation was found between the total SOST amount and OPG in all groups ($P > .05$, $r = -.128$). Additionally, significant low negative relationships were identified between CAL ($P < .05$, $r=-0.261$) and PD ($P < .05$, $r=-0.296$), and OPG.

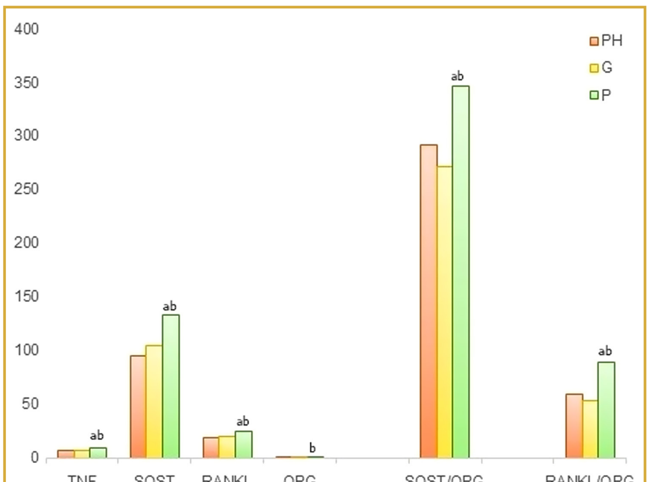

Figure 1. Salivary tumor necrosis factor alpha (TNF-α), sclerostin (SOST), receptor activator nuclear factor-κB ligand (RANKL), osteoprotogerin levels in the study groups. Significant differences between the groups ($P < .05$ by Kruskal-Wallis test). [a]Significantly higher than Periodontal health group. [b]Significantly different from Gingivitis group. [ab]Significantly different from the Periodontal health and Gingivitis groups. Periodontitis group exhibited the highest biochemical parameters levels when compared to the other groups. Data are expressed as mean ± SD.

Receiver Operating Characteristic Curve Analysis

Figure 2 displays the ROC and AUC curves utilized to evaluate the diagnostic sensitivity and specificity of the examined salivary biomarkers. The markers TNF-α, RANKL, and SOST demonstrated the greatest sensitivity and specificity when comparing the groups of individuals with periodontal disease, either combined or separately, to the groups of individuals with healthy periodontal conditions. TNF-α exhibited the greatest sensitivity and specificity in distinguishing between patients with periodontitis and individuals who are periodontally healthy, with RANKL and SOST following closely behind. Comparing the periodontitis group with other groups, the AUC values of TNF-α, RANKL, and SOST were 0.915, 0.917, and 0.800, respectively.

DISCUSSION

To the best of our knowledge, the current research represents the first clinical trial that examines salivary SOST levels in subjects with different periodontal conditions and investigates the correlation between the salivary RANKL/OPG ratio and SOST/OPG in subjects. In periodontal disease, the local cellular response in the gingiva is reflected by salivary components. Saliva is easily accessible by a completely noninvasive collection method, and saliva includes locally generated microbial and host response mediators.[23] Recently, saliva has been used to predict caries risk,[24] oral cancer, salivary gland diseases, hepatitis, human immunodeficiency virus (HIV), hepatitis C virus, and periodontitis.[25] This study evaluated markers of bone metabolism in saliva samples from patients with periodontal disease.

The fragile balance between osteoclasts and osteoblasts controls bone metabolism. In periodontal disease, gingival cells are directly involved in triggering the host response, which disrupts the homeostatic mechanism and remodeling of the bone matrix.[19] Receptor activator of NF-κB/RANKL/OPG, with canonical (Wnt)/β-catenin signaling, are key regulators of bone remodeling.[26,27] The literature has demonstrated that the expression of RANK and RANKL is upregulated in areas with severe chronic periodontitis, suggesting their role in the pathology that underlies periodontitis. Studies have reported that SOST, which represents a glycoprotein product of the SOST gene, is synthesized by differentiated cells, e.g., chondrocyte, osteocytes, and cementocyte.[10] Sclerostin is a biomarker that negatively controls the development of new bone by inhibiting the WNT signaling pathway and osteoblastogenesis.[8,10] Suppressed SOST levels led to elevated bone formation and mineral density, bone strength, and bone volume in mice.[28] A study on ligature-induced periodontitis

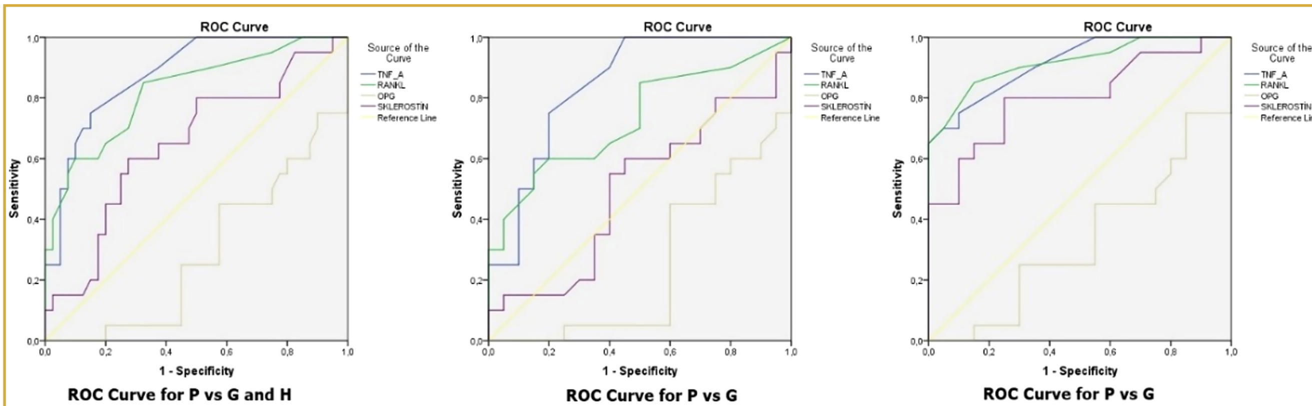

Figure 2. Receiver operating characteristic analysis of TNF-α, RANKL, sclerostin, and OPG. G, gingivitis; P, periodontitis; H, periodontally healthy.

in rats showed elevated osteocytic SOST expression, which was associated with the suppression of osteoid formation. Elevated expression of RANKL (increased formation of osteoclasts) and SOST (suppressed formation of osteoids) has been reported as evidence of alveolar bone loss in ligature-induced periodontal disease.[29] A recent report found higher SOST levels in subjects with peri-implantitis than in subjects with peri-mucositis and healthy peri-implant tissues.[30] A clinical trial investigating the effect of SOST on periodontitis found higher SOST levels in GCF samples of individuals with chronic periodontitis and reported a positive correlation between SOST levels and PD and CAL.[14]

Mechanosensors, cytokines, and endocrine factors regulate SOST expression.[31] Research examining SOST levels in subjects with periodontitis has found higher SOST levels in GCF and PICF specimens than in healthy controls.[31] Napimoga et al.[14] reported elevated mRNA levels of SOST, TNF-α, and Dickkopf-related protein 1 (DKK1) in tissues and increased serum protein levels of TNF-α and SOST. Ballı et al.[2] reported increased GCF SOST levels in subjects with periodontitis and identified decreased SOST concentrations following nonsurgical periodontal treatment. Considering that the potential role of SOST in bone metabolism and salivary SOST levels in subjects with periodontitis have not been evaluated before, the current study compared salivary SOST levels in subjects with chronic periodontitis, subjects with gingivitis, and healthy individuals. The data provided in our study support the findings of other studies that have shown higher levels of SOST and RANKL/OPG ratios in saliva samples obtained from subjects with periodontitis compared with healthy individuals. The above-mentioned values suggest that SOST levels indicate the pathogenesis of the disease in a reliable manner.[2]

Receptor activator of NF-κB ligand and SOSLexpression in osteocytes is stimulated by proinflammatory cytokines, including TNF-α and IL-1β. An in vitro study demonstrated that the expression of RANKL by the osteocyte-like MYO-Y4 cell line was elevated by IL-1β, which improved MLOY4-mediated osteoclastogenesis.[32] Another group stated that TNF-α induced the expression of SOST in osteocytes in vitro and increased the expression of SOST in osteocytes of osteopenic TNF transgenic mice.[33] These findings show that inflammatory responses can contributite to elevated bone resorption and decreased bone formation by stimulating the expression of RANKL and SOST in osteocytes. The research carried out by Reis et al.[34] found significantly higher TNF-α levels in diseased regions than in non-diseased regions. Gokul et al.[35] compared subjects with chronic periodontitis, gingivitis, and healthy subjects and showed significantly higher TNF-α levels in patients with gingivitis and chronic periodontitis in comparison with healthy individuals, whereas no significant difference was found between subjects with gingivitis and periodontitis. In this study, salivary levels of TNF-α were shown to be elevated in periodontitis, gingivitis, and healthy subjects compared with subjects with and without periodontitis. It is possible to explain the relationship between periodontal status and TNF-α levels by the bone destruction observed in periodontitis. However, further research on the relationship between proinflammatory cytokines and periodontitis is required to elucidate the mechanism of RANKL and SOST expression in periodontitis.

CONCLUSION

This study evaluated the impact of WNT signaling on periodontal disease and investigated whether salivary. Sclerostin, RANKL/OPG, and TNF-α as diagnostic markers of periodontal status. Within the limitations of this study, high salivary SOST levels in periodontitis and their positive correlations with TNF-α and bone markers suggest that these molecules are involved in the pathogenesis of periodontal disease. Future studies aimed at investigating the potential of SOST as a reliable biomarker in periodontal disease could broaden the horizons of periodontal diagnostic tools.

Availability of Data and Materials: The data that support the findings of this study are available on request from the corresponding author.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Necmettin Erbakan University (Approval no: 2021/05-62, Date: May 11, 2021).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – F.U.Y., Ü.T.A.; Design – F.U.Y.; Data Collection and/or Processing – F.U.Y., Ü.T.A.; Analysis and/or Interpretation – F.U.Y., Ü.T.A., F.A.; Writing Manuscript – F.U.Y.; Critical Review – F.U.Y., Ü.T.A., F.A.

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EVALUATION OF BIOMARKERS IN PATIENTS WITH DIFFERENT PERIODONTAL STATUSES

STROBE STATEMENT—CHECKLIST OF ITEMS THAT SHOULD BE INCLUDED IN REPORTS OF OBSERVATIONAL STUDIES

	Item No.	Recommendation	Page No.
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	1
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	2
Objectives	3	State specific objectives, including any prespecified hypotheses	3
Methods			
Study design	4	Present key elements of study design early in the paper	4-5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	4-5
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up. *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls. *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	4
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed. *Case-control study*—For matched studies, give matching criteria and the number of controls per case	4
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	4-5
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe the comparability of assessment methods if there is more than one group	*5*
Bias	9	Describe any efforts to address potential sources of bias	N/A
Study size	10	Explain how the study size was arrived at	N/A
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	5
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	5
		(*b*) Describe any methods used to examine subgroups and interactions	N/A
		(*c*) Explain how missing data were addressed	N/A
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed. *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking into account of sampling strategy	N/A
		(*e*) Describe any sensitivity analyses	N/A
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	5-6
		(b) Give reasons for non-participation at each stage	N/A
		(c) Consider use of a flow diagram	N/A
Descriptive data	14*	(a) Give characteristics of study participants (e.g., demographic, clinical, social) and information on exposures and potential confounders	3-4
		(b) Indicate number of participants with missing data for each variable of interest	N/A
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	N/A
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	
		Cross-sectional study—Report numbers of outcome events or summary measures	5-6

	Item No.	Recommendation	Page No.
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	N/A
		(*b*) Report category boundaries when continuous variables were categorized	5
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	N/A
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	5
Discussion			
Key results	18	Summarise key results with reference to study objectives	6,8
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	N/A
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	6-8
Generalisability	21	Discuss the generalizability (external validity) of the study results	6-8
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	1

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the websites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.